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(54) Title: METHOD FOR THE TREATMENT OF GRAFTS

(57) Abstract

The present invention provides a method for treatment of grafts which comprises introducing a nucleic acid encoding an angiogenic agent into the cells of the graft. The graft may be treated *ex vivo* and then transplanted into the donor or may be treated after transplantation. The graft may be autologous, allogenic, xenogenic or a tissue engineered graft ("bio–artifical" organ). The nucleic acid may be introduced to the cultured cells used to form the tissue engineered graft. Expression of the angiogenic agent by the cells of the graft promotes growth of new blood vessels (angiogenesis) providing the graft with a blood supply thus increasing the chances for graft survival.

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METHOD FOR THE TREATMENT OF GRAFTS

BACKGROUND OF THE INVENTION

The success of a transplant of an allograft in a host depends on such factors as the antigens on the transplanted tissue that are recognized by the recipient as foreign and can evoke the rejection response, the cells in the recipient's immune system that mediate rejection, and the reactions that modify either the presentation of the foreign antigen or the cellular response.

While the immunological response to transplant tissue may be suppressed through the use of immunosuppressant drugs to minimize tissue rejection, immunosuppressant therapy is general in nature. Hence, immunosuppressant drugs tend to suppress the immune response, which reduces the transplant patient's ability to combat infection.

In view of these complications, transplantation immunologists have sought methods for suppressing immune responsiveness in an antigen-specific manner (so that only the response to the donor alloantigen would be lost). For example, the survival time of skin grafts has been prolonged by a factor of two by treatment in vitro with cortisone, thalidomide, or urethane before implantation into a laboratory animal. The amount of drug locally applied to the skin was smaller than the amount required to achieve a similar effect by injecting the drug systemically. In an additional study, the donor skin was treated in vitro with streptokinase/streptodornase, or with RNA and DNA preparations of the recipient. Further, treatment of transplant tissues with a solution of glutaraldehyde prior to transplantation was found to reduce their antigenicity. See U.S. Pat. No. 4,120,649.

TGF-beta has been found to suppress the expression of Class II histocompatibility antigens on human cells induced by human interferon-gamma and to inhibit constitutive expression of the Class II antigen message in the cells. Use of recombinant TGF-beta as an immunosuppressive agent for the treatment of graphs prior to implantation has been proposed. See U.S. Pat. No. 5,135,915.

In addition to immune rejection, many grafts, especially skin grafts, fail

due to lack of suitable blood supply. This is particularly true in patients in whom ulcers develop in the setting of limb ischemia.

It would be desirable to have a method to prolong graft survival in transplant operations that minimizes the toxicity and other adverse effects arising from the use of large doses of immunosuppressants.

It would further be desirable to have a method to prolong graft survival and wound healing in the treatment of skin ulcers in patients with limb ischemia.

SUMMARY OF THE INVENTION

The present invention provides a method for treatment of grafts which comprises introducing a nucleic acid encoding an angiogenic agent into the cells of the graft. The graft may be treated *ex vivo* and then transplanted into the donor or may be treated after transplantation. The graft may be autologous, allogenic, xenogenic or a tissue engineered graft ("bio-artifical" organ). The nucleic acid may be introduced to the cultured cells used to form the tissue engineered graft. Expression of the angiogenic agent by the cells of the graft promotes growth of new blood vessels (angiogenesis) providing the graft with a blood supply thus increasing the chances for graft survival.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph of the mouse model used in which a full thickness skin wound was created in the dorsal integument overlying the upper spine.

Figure 2 shows the gross appearance of a graft that has been placed over the wound shown in Figure 1. In this case the graft was generated by transfecting keratinocytes in culture with the adenovirus construct encoding beta-galactosidase. After the keratinocytes had been incorporated into the skin used to perform the grafting, the graft was allowed to survive for 7 days at which time it was removed along with a border of normal skin and then stained with X-Gal to identify the staining related to the expression of beta-

galactosidase in the keratinocytes of the graft.

Figures 3A-3C shows photomicrographs of a control graft (3A) that was prepared from keratinocytes that were not transfected with beta galactosidase. Figures 3B and 3C show grafts that were prepared from keratinocytes that had been transduced with the adenovirus beta-glactosidase construct (driven by the cytomegalovirus promoter) at multiplicity of infections of 37 (3B) and 150 (3C). The dark staining identifies keratinocytes which are actively expressing the *lacZ* transgene encoding for beta-glactosidase.

DETAILED DESCRIPTION OF THE INVENTION

The term "graft" as used herein refers to biological material derived from a donor for transplantation into a recipient. Grafts include tissues and organs in which would benefit from vascularization. Organs include, for example, skin, heart, liver, spleen, pancreas, thyroid lobe, lung, kidney, tubular organs (e.g., intestine, blood vessels, or esophagus), etc. The tubular organs can be used to replace damaged portions of esophagus, blood vessels, or bile duct. The skin grafts can be used not only for ischemic skin ulcers and burns, but also as a dressing to damaged intestine or to close certain defects such as diaphragmatic hernia. The graft is derived from any source, preferably mammalian, including human, whether from cadavers or living donors. Alternatively, the graft may be a tissue engineered graft formed from a combination of cultured cells and scaffold material. An example of such a tissue engineered graft is Appligraf ®. Appligraf ® consists of a type I collagen gel seeded with allogenic fibroblasts covered with a confluent surface layer of allogenic keratinocytes.

The term "host" as used herein refers to any compatible transplant recipient. By "compatible" is meant a host that will accept the donated graft. Preferably, the host is mammalian, and more preferably human. If both the donor of the graft and the host are human, they are preferably matched for HLA class II antigens so as to improve histocompatibility.

The term "donor" as used herein refers to the species, dead or alive, from which the graft is derived. Preferably, the donor is mammalian. Human donors are preferably volunteer blood-related donors that are normal on physical

examination and of the same major ABO blood group, because crossing major blood group barriers possibly prejudices survival of the allograft. It is, however, possible to transplant, for example, a kidney of a type O donor into an A, B or AB recipient.

The terms "transplant" and "implant" are used interchangeably to refer to tissue or cells (xenogeneic or allogeneic) which may be introduced into the body of a host to replace or structure or function of the endogenous tissue.

The term "angiogenic agent" refers to any protein, polypeptide, mutein or portion that is capable of, directly or indirectly, inducing the formation of new blood vessels. Folkman, et al., *Science*, 235:442-447 (1987). Such proteins include, for example, acidic fibroblast growth factors (FGF-1), basic fibroblast growth factors (FGF-2)), FGF-4, FGF-5, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF-α and TFG-β), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNF-α), hepatocyte growth factor (HGF, scatter factor), insulin like growth factor (IGF), IL-8, proliferin, angiogenin, fibrin fragment E, angiotropin, erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF) and nitric oxidesynthase (NOS). VEGF includes the various forms of VEGF such as $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, and $VEGF_{189}$. See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217-239 (1991); Folkman, et al., *J. Biol. Chem.*, 267:10931-10934 (1992) and Symes, et al., *Current Opinion in Lipidology*, 5:305-312 (1994).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature*, 362:844 (1993).

The angiogenic action of any given protein, peptide or mutein can be determined using a number of bioassays including, for example, the rabbit cornea pocket assay (Gaudric et al., *Ophthalmic. Res.* 24:181-8 (1992)) and the

chicken chorioallantoic membrane (CAM) assay (Peek et al., *Exp. Pathol.* 34:35-40 (1988)).

The nucleotide sequence of numerous angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the nucleic acid encoding the protein, the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter must be capable of driving expression of the protein in cells of the desired target tissue. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., *Hum Gene Ther* 4:151 (1993)) and MMT promoters may also be used. Certain proteins can expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a *tat* gene and *tar* element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the β-lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

In certain situations, it may be desirable to use nucleic acid's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene

products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-arginine, fibronectin, urokinase, plasminogen activator and heparin.

The term "effective amount" means a sufficient amount of nucleic acid delivered to produce an adequate level of the angiogenic protein, i.e., levels capable of inducing angiogenesis. Thus, the important aspect is the level of protein expressed. Accordingly, one can use multiple transcripts or one can have the gene under the control of a promoter that will result in high levels of expression. In an alternative embodiment, the gene would be under the control of a factor that results in extremely high levels of expression, e.g., *tat* and the corresponding *tar* element.

Typically, the nucleic acid encoding the angiogenic agent is formulated by mixing it at ambient temperature at the appropriate pH, and at the desired degree of purity, with physiologically acceptable carriers, i.e., carriers that are non-toxic to recipients at the dosages and concentrations employed.

The nucleic acids are introduced into the cells of the graft by any method which will result in the uptake and expression of the nucleic acid by the cells. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, $Ca_3(PO_4)_2$ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, viral vectors, adjuvant-assisted DNA, catheters, gene guns etc. Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox

or avipox vectors, herpes virus vectors such as a herpes simplex I virus (HSV) vector [A.I. Geller et al., *J. Neurochem*, 64:487 (1995); F. Lim et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); A.I. Geller et al., *Proc Natl. Acad. Sci.*: U.S.A.:90 7603 (1993); A.I. Geller et al., *Proc Natl. Acad. Sci USA*: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., Nat. Genet., 3:219 (1993); Yang et al., *J. Virol.*, 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al., *Nat. Genet.*, 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated.

Gene guns include those disclosed in U.S. Patent Numbers 5,100,792 and 5,371,015 and PCT publication WO 91/07487.

If desired, the nucleic acid may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, *BioTechniques*, 6:682 (1988). See also, Felgner and Holm, *Bethesda Res. Lab. Focus*, 11(2):21 (1989) and Maurer, R.A., *Bethesda Res. Lab. Focus*, 11(2):25 (1989).

Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., *Proc. Natl. Acad. Sci. USA*, 89:2581-2584 (1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

For delivery of the nucleic acid to a skin graft, the graft may submerged in the nucleic acid composition for a sufficient time to allow up take of the nucleic acid.

For use in tissue engineered grafts, the cells used to form the graft are

transfected with the nucleic acid encoding the angiogenic agent. Preferably, the cells are transfected prior to formation of the graft. For example with a tissue engineered graft such as a synthetic skin equivalent, e.g., Apligraph® (Organogenesis, Canton, MA.) the keratinocytes used to form the graft can be transfected in culture with a vector containing a DNA encoding the angiogenic agent.

The nucleic acid may be introduced by direct injection into the graft prior to, or after, transplantation.

The nucleic acid can be applied topically, for example, painted onto a skin graft prior to transplantation. In such a case it is preferable to use a viscous solution such as a gel rather than a non-viscous solution. This may be accomplished, for example, by mixing the solution of the nucleic acid with a gelling agent, such as a polysaccharide, preferably a water-soluble polysaccharide, such as, e.g., hyaluronic acid, starches, and cellulose derivatives, e.g., methylcellulose, hydroxyethyl cellulose, and carboxymethyl cellulose. The most preferred gelling agent is methylcellulose. The polysaccharide is generally present in a gel formulation in the range of 1-90% by weight of the gel, more preferably 1-20%. Examples of other suitable polysaccharides for this purpose, and a determination of the solubility of the polysaccharides, are found in EP 267,015, published May 11, 1988, the disclosure of which is incorporated herein by reference.

In certain situations the nucleic acid is introduced by contacting the graft the nucleic acid in an appropriate composition. The contact suitably involves incubating or perfusing the organ with the composition or applying the composition to one or more surfaces of the graft for a sufficient time to allow the nucleic acid to be taken up by the cells of the graft. The treatment generally takes place for at least one minute, and preferably from 1 minute to 72 hours, and more preferably from 2 minutes to 24 hours, depending on such factors as the concentration of nucleic acid in the formulation, the graft to be treated, and the particular type of formulation. Perfusion is accomplished by any suitable procedure. For example, an organ can be perfused via a device that provides a constant pressure of perfusion having a pressure regulator and overflow

situated between a pump and the organ, as described by DD 213,134 published Sep. 5, 1984. Alternatively, the organ is placed in a hyperbaric chamber via a sealing door and perfusate is delivered to the chamber by a pump that draws the fluid from the reservoir while spent perfusate is returned to the reservoir by a valve, as described in EP 125,847 published Nov. 21, 1984.

Prior to transplantation, the host can be treated pre-transplant procedures that would be beneficial to the particular transplant recipient.

The transplantation procedure itself will depend on the particular disorder being treated, the condition of the patient, etc. The medical practitioner will recognize the appropriate procedure to employ in any given case. The transplants are optionally monitored systematically during the critical postoperative period (the first three months) using any suitable procedure. After the transplantation, immunosuppression therapy may be utilized as necessary to ensure graft survival.

What is claimed is:

1. A method for the treatment of a graft comprising, introducing an effective amount of a nucleic acid encoding an angiogenic agent to the cells of the graft.

2. The method of claim 1, wherein the nucleic acid is contacted prior to transplantation of the graft into a compatible host.

3. The method of claim 1, wherein the graft is tissue.

4. The method of claim 1, wherein the graft is skin.

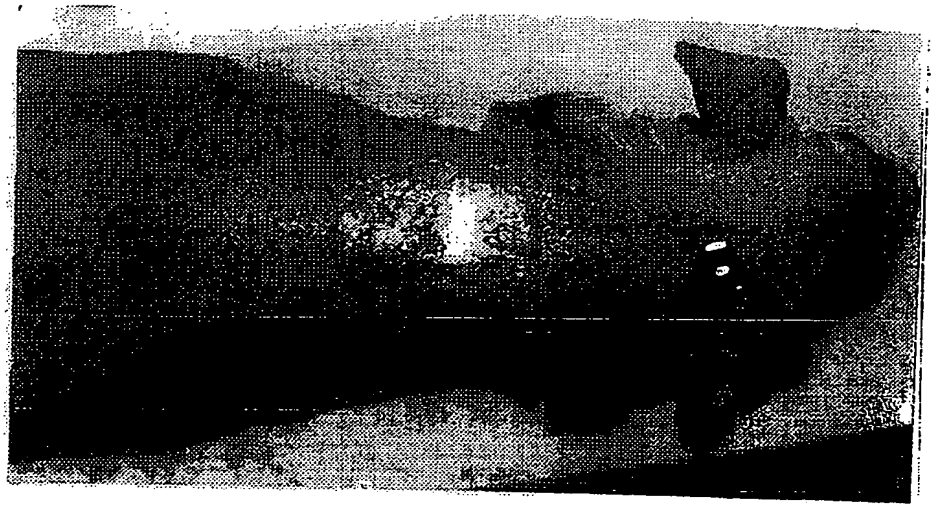

FIG. 1

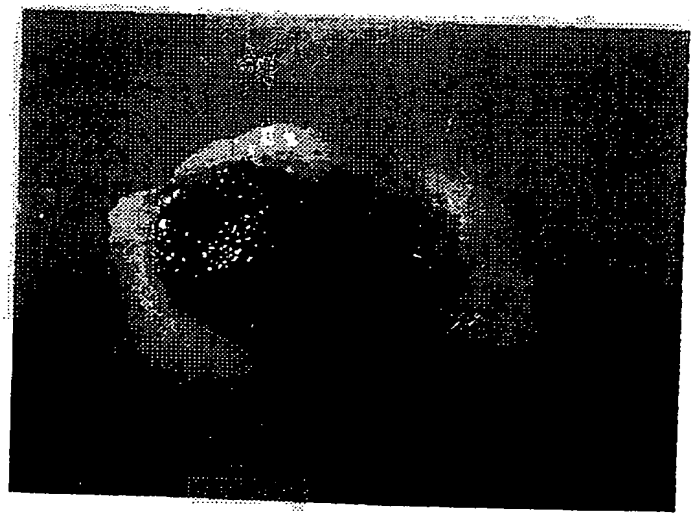

FIG. 2

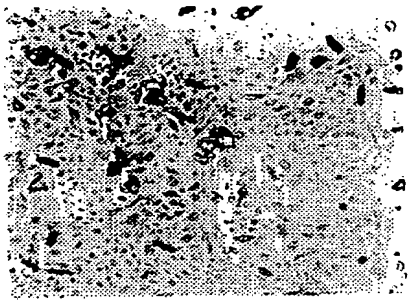

FIG. 3A

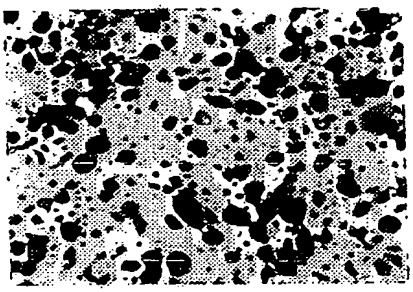

FIG. 3B

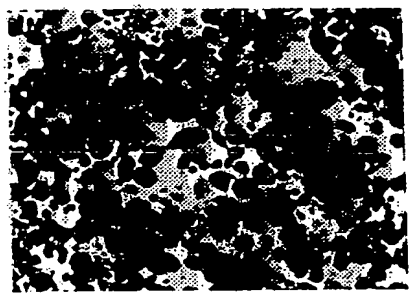

FIG. 3C

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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US CL :514/44; 424/93.2, 93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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APS and Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NABEL, E. et al. Recombinant Fibroblast Growth Factor-1 Promotes Intimal Hyperplasia and Angiogenesis in Arteries In Vivo. Nature. 29 April 1993, Vol. 362, pages 844-846, see entire document.	1-4
Y	DETMAR, M. et al. Overexpression of Vascular Permeability Factor/Vascular Endothelial Growth Factor and Its Receptors in Psoriasis. Journal of Experimental Medicine. September 1994, Vol. 180, pages 1141-1146, see entire document.	1-4
Y	US 5,639,725 A (O'REILLY et al.) 17 June 1997, col. 4, lines 21-47.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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(57) Abstract

The present invention provides a method for treatment of grafts which comprises introducing a nucleic acid encoding an angiogenic agent into the cells of the graft. The graft may be treated *ex vivo* and then transplanted into the donor or may be treated after transplantation. The graft may be autologous, allogenic, xenogenic or a tissue engineered graft ("bio-artifical" organ). The nucleic acid may be introduced to the cultured cells used to form the tissue engineered graft. Expression of the angiogenic agent by the cells of the graft promotes growth of new blood vessels (angiogenesis) providing the graft with a blood supply thus increasing the chances for graft survival.

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METHOD FOR THE TREATMENT OF GRAFTS

BACKGROUND OF THE INVENTION

The success of a transplant of an allograft in a host depends on such factors as the antigens on the transplanted tissue that are recognized by the recipient as foreign and can evoke the rejection response, the cells in the recipient's immune system that mediate rejection, and the reactions that modify either the presentation of the foreign antigen or the cellular response.

While the immunological response to transplant tissue may be suppressed through the use of immunosuppressant drugs to minimize tissue rejection, immunosuppressant therapy is general in nature. Hence, immunosuppressant drugs tend to suppress the immune response, which reduces the transplant patient's ability to combat infection.

In view of these complications, transplantation immunologists have sought methods for suppressing immune responsiveness in an antigen-specific manner (so that only the response to the donor alloantigen would be lost). For example, the survival time of skin grafts has been prolonged by a factor of two by treatment in vitro with cortisone, thalidomide, or urethane before implantation into a laboratory animal. The amount of drug locally applied to the skin was smaller than the amount required to achieve a similar effect by injecting the drug systemically. In an additional study, the donor skin was treated in vitro with streptokinase/streptodornase, or with RNA and DNA preparations of the recipient. Further, treatment of transplant tissues with a solution of glutaraldehyde prior to transplantation was found to reduce their antigenicity. See U.S. Pat. No. 4,120,649.

TGF-beta has been found to suppress the expression of Class II histocompatibility antigens on human cells induced by human interferon-gamma and to inhibit constitutive expression of the Class II antigen message in the cells. Use of recombinant TGF-beta as an immunosuppressive agent for the treatment of graphs prior to implantation has been proposed. See U.S. Pat. No. 5,135,915.

In addition to immune rejection, many grafts, especially skin grafts, fail

due to lack of suitable blood supply. This is particularly true in patients in whom ulcers develop in the setting of limb ischemia.

It would be desirable to have a method to prolong graft survival in transplant operations that minimizes the toxicity and other adverse effects arising from the use of large doses of immunosuppressants.

It would further be desirable to have a method to prolong graft survival and wound healing in the treatment of skin ulcers in patients with limb ischemia.

SUMMARY OF THE INVENTION

The present invention provides a method for treatment of grafts which comprises introducing a nucleic acid encoding an angiogenic agent into the cells of the graft. The graft may be treated *ex vivo* and then transplanted into the donor or may be treated after transplantation. The graft may be autologous, allogenic, xenogenic or a tissue engineered graft ("bio-artifical" organ). The nucleic acid may be introduced to the cultured cells used to form the tissue engineered graft. Expression of the angiogenic agent by the cells of the graft promotes growth of new blood vessels (angiogenesis) providing the graft with a blood supply thus increasing the chances for graft survival.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photograph of the mouse model used in which a full thickness skin wound was created in the dorsal integument overlying the upper spine.

Figure 2 shows the gross appearance of a graft that has been placed over the wound shown in Figure 1. In this case the graft was generated by transfecting keratinocytes in culture with the adenovirus construct encoding beta-galactosidase. After the keratinocytes had been incorporated into the skin used to perform the grafting, the graft was allowed to survive for 7 days at which time it was removed along with a border of normal skin and then stained with X-Gal to identify the staining related to the expression of beta-

galactosidase in the keratinocytes of the graft.

Figures 3A-3C shows photomicrographs of a control graft (3A) that was prepared from keratinocytes that were not transfected with beta galactosidase. Figures 3B and 3C show grafts that were prepared from keratinocytes that had been transduced with the adenovirus beta-glactosidase construct (driven by the cytomegalovirus promoter) at multiplicity of infections of 37 (3B) and 150 (3C). The dark staining identifies keratinocytes which are actively expressing the *lacZ* transgene encoding for beta-glactosidase.

DETAILED DESCRIPTION OF THE INVENTION

The term "graft" as used herein refers to biological material derived from a donor for transplantation into a recipient. Grafts include tissues and organs in which would benefit from vascularization. Organs include, for example, skin, heart, liver, spleen, pancreas, thyroid lobe, lung, kidney, tubular organs (e.g., intestine, blood vessels, or esophagus), etc. The tubular organs can be used to replace damaged portions of esophagus, blood vessels, or bile duct. The skin grafts can be used not only for ischemic skin ulcers and burns, but also as a dressing to damaged intestine or to close certain defects such as diaphragmatic hernia. The graft is derived from any source, preferably mammalian, including human, whether from cadavers or living donors. Alternatively, the graft may be a tissue engineered graft formed from a combination of cultured cells and scaffold material. An example of such a tissue engineered graft is Appligraf ®. Appligraf ® consists of a type I collagen gel seeded with allogenic fibroblasts covered with a confluent surface layer of allogenic keratinocytes.

The term "host" as used herein refers to any compatible transplant recipient. By "compatible" is meant a host that will accept the donated graft. Preferably, the host is mammalian, and more preferably human. If both the donor of the graft and the host are human, they are preferably matched for HLA class II antigens so as to improve histocompatibility.

The term "donor" as used herein refers to the species, dead or alive, from which the graft is derived. Preferably, the donor is mammalian. Human donors are preferably volunteer blood-related donors that are normal on physical

examination and of the same major ABO blood group, because crossing major blood group barriers possibly prejudices survival of the allograft. It is, however, possible to transplant, for example, a kidney of a type O donor into an A, B or AB recipient.

The terms "transplant" and "implant" are used interchangeably to refer to tissue or cells (xenogeneic or allogeneic) which may be introduced into the body of a host to replace or structure or function of the endogenous tissue.

The term "angiogenic agent" refers to any protein, polypeptide, mutein or portion that is capable of, directly or indirectly, inducing the formation of new blood vessels. Folkman, et al., *Science*, 235:442-447 (1987). Such proteins include, for example, acidic fibroblast growth factors (FGF-1), basic fibroblast growth factors (FGF-2)), FGF-4, FGF-5, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor α and β (TGF-α and TFG-β), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor α (TNF-α), hepatocyte growth factor (HGF, scatter factor), insulin like growth factor (IGF), IL-8, proliferin, angiogenin, fibrin fragment E, angiotropin, erythropoietin, colony stimulating factor (CSF), macrophage-CSF (M-CSF), granulocyte/macrophage CSF (GM-CSF) and nitric oxidesynthase (NOS). VEGF includes the various forms of VEGF such as $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, and $VEGF_{189}$. See, Klagsbrun, et al., *Annu. Rev. Physiol.*, 53:217-239 (1991); Folkman, et al., *J. Biol. Chem.*, 267:10931-10934 (1992) and Symes, et al., *Current Opinion in Lipidology*, 5:305-312 (1994).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., *Nature*, 362:844 (1993).

The angiogenic action of any given protein, peptide or mutein can be determined using a number of bioassays including, for example, the rabbit cornea pocket assay (Gaudric et al., *Ophthalmic. Res.* 24:181-8 (1992)) and the

chicken chorioallantoic membrane (CAM) assay (Peek et al., *Exp. Pathol.* 34:35-40 (1988)).

The nucleotide sequence of numerous angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the nucleic acid encoding the protein, the nucleic acid is preferably inserted into a cassette where it is operably linked to a promoter. The promoter must be capable of driving expression of the protein in cells of the desired target tissue. The selection of appropriate promoters can readily be accomplished. Preferably, one would use a high expression promoter. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, et al., *Hum Gene Ther* 4:151 (1993)) and MMT promoters may also be used. Certain proteins can expressed using their native promoter. Other elements that can enhance expression can also be included such as an enhancer or a system that results in high levels of expression such as a *tat* gene and *tar* element. This cassette can then be inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an *E. coli* origin of replication. See, Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the β-lactamase gene for ampicillin resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. The cassette can also be bound to a nucleic acid binding moiety in a synthetic delivery system, such as the system disclosed in WO 95/22618.

In certain situations, it may be desirable to use nucleic acid's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene

products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-arginine, fibronectin, urokinase, plasminogen activator and heparin.

The term "effective amount" means a sufficient amount of nucleic acid delivered to produce an adequate level of the angiogenic protein, i.e., levels capable of inducing angiogenesis. Thus, the important aspect is the level of protein expressed. Accordingly, one can use multiple transcripts or one can have the gene under the control of a promoter that will result in high levels of expression. In an alternative embodiment, the gene would be under the control of a factor that results in extremely high levels of expression, e.g., *tat* and the corresponding *tar* element.

Typically, the nucleic acid encoding the angiogenic agent is formulated by mixing it at ambient temperature at the appropriate pH, and at the desired degree of purity, with physiologically acceptable carriers, i.e., carriers that are non-toxic to recipients at the dosages and concentrations employed.

The nucleic acids are introduced into the cells of the graft by any method which will result in the uptake and expression of the nucleic acid by the cells. The introduction can be by standard techniques, e.g. infection, transfection, transduction or transformation. Examples of modes of gene transfer include e.g., naked DNA, $Ca_3(PO_4)_2$ precipitation, DEAE dextran, electroporation, protoplast fusion, lipofecton, cell microinjection, viral vectors, adjuvant-assisted DNA, catheters, gene guns etc. Vectors include chemical conjugates such as described in WO 93/04701, which has targeting moiety (e.g. a ligand to a cellular surface receptor), and a nucleic acid binding moiety (e.g. polylysine), viral vector (e.g. a DNA or RNA viral vector), fusion proteins such as described in PCT/US 95/02140 (WO 95/22618) which is a fusion protein containing a target moiety (e.g. an antibody specific for a target cell) and a nucleic acid binding moiety (e.g. a protamine), plasmids, phage, etc. The vectors can be chromosomal, non-chromosomal or synthetic.

Preferred vectors include viral vectors, fusion proteins and chemical conjugates. Retroviral vectors include moloney murine leukemia viruses. DNA viral vectors are preferred. These vectors include pox vectors such as orthopox

or avipox vectors, herpes virus vectors such as a herpes simplex I virus (HSV) vector [A.I. Geller et al., *J. Neurochem*, 64:487 (1995); F. Lim et al., in *DNA Cloning: Mammalian Systems*, D. Glover, Ed. (Oxford Univ. Press, Oxford England) (1995); A.I. Geller et al., *Proc Natl. Acad. Sci.*: U.S.A.:90 7603 (1993); A.I. Geller et al., *Proc Natl. Acad. Sci USA*: 87:1149 (1990)], Adenovirus Vectors [LeGal LaSalle et al., *Science*, 259:988 (1993); Davidson, et al., Nat. Genet., 3:219 (1993); Yang et al., *J. Virol.*, 69: 2004 (1995)] and Adeno-associated Virus Vectors [Kaplitt, M.G., et al., *Nat. Genet.*, 8:148 (1994)].

Pox viral vectors introduce the gene into the cells cytoplasm. Avipox virus vectors result in only a short term expression of the nucleic acid. Adenovirus vectors, adeno-associated virus vectors and herpes simplex virus (HSV) vectors are preferred for introducing the nucleic acid into neural cells. The adenovirus vector results in a shorter term expression (about 2 months) than adeno-associated virus (about 4 months), which in turn is shorter than HSV vectors. The particular vector chosen will depend upon the target cell and the condition being treated.

Gene guns include those disclosed in U.S. Patent Numbers 5,100,792 and 5,371,015 and PCT publication WO 91/07487.

If desired, the nucleic acid may also be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, *BioTechniques*, 6:682 (1988). See also, Felgner and Holm, *Bethesda Res. Lab. Focus*, 11(2):21 (1989) and Maurer, R.A., *Bethesda Res. Lab. Focus*, 11(2):25 (1989).

Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., *Proc. Natl. Acad. Sci. USA*, 89:2581-2584 (1992); Stratford-Perricadet, et al., *J. Clin. Invest.*, 90:626-630 (1992); and Rosenfeld, et al., *Cell*, 68:143-155 (1992).

For delivery of the nucleic acid to a skin graft, the graft may submerged in the nucleic acid composition for a sufficient time to allow up take of the nucleic acid.

For use in tissue engineered grafts, the cells used to form the graft are

transfected with the nucleic acid encoding the angiogenic agent. Preferably, the cells are transfected prior to formation of the graft. For example with a tissue engineered graft such as a synthetic skin equivalent, e.g., Apligraph® (Organogenesis, Canton, MA.) the keratinocytes used to form the graft can be transfected in culture with a vector containing a DNA encoding the angiogenic agent.

The nucleic acid may be introduced by direct injection into the graft prior to, or after, transplantation.

The nucleic acid can be applied topically, for example, painted onto a skin graft prior to transplantation. In such a case it is preferable to use a viscous solution such as a gel rather than a non-viscous solution. This may be accomplished, for example, by mixing the solution of the nucleic acid with a gelling agent, such as a polysaccharide, preferably a water-soluble polysaccharide, such as, e.g., hyaluronic acid, starches, and cellulose derivatives, e.g., methylcellulose, hydroxyethyl cellulose, and carboxymethyl cellulose. The most preferred gelling agent is methylcellulose. The polysaccharide is generally present in a gel formulation in the range of 1-90% by weight of the gel, more preferably 1-20%. Examples of other suitable polysaccharides for this purpose, and a determination of the solubility of the polysaccharides, are found in EP 267,015, published May 11, 1988, the disclosure of which is incorporated herein by reference.

In certain situations the nucleic acid is introduced by contacting the graft the nucleic acid in an appropriate composition. The contact suitably involves incubating or perfusing the organ with the composition or applying the composition to one or more surfaces of the graft for a sufficient time to allow the nucleic acid to be taken up by the cells of the graft. The treatment generally takes place for at least one minute, and preferably from 1 minute to 72 hours, and more preferably from 2 minutes to 24 hours, depending on such factors as the concentration of nucleic acid in the formulation, the graft to be treated, and the particular type of formulation. Perfusion is accomplished by any suitable procedure. For example, an organ can be perfused via a device that provides a constant pressure of perfusion having a pressure regulator and overflow

situated between a pump and the organ, as described by DD 213,134 published Sep. 5, 1984. Alternatively, the organ is placed in a hyperbaric chamber via a sealing door and perfusate is delivered to the chamber by a pump that draws the fluid from the reservoir while spent perfusate is returned to the reservoir by a valve, as described in EP 125,847 published Nov. 21, 1984.

Prior to transplantation, the host can be treated pre-transplant procedures that would be beneficial to the particular transplant recipient.

The transplantation procedure itself will depend on the particular disorder being treated, the condition of the patient, etc. The medical practitioner will recognize the appropriate procedure to employ in any given case. The transplants are optionally monitored systematically during the critical postoperative period (the first three months) using any suitable procedure. After the transplantation, immunosuppression therapy may be utilized as necessary to ensure graft survival.

What is claimed is:

1. A method for the treatment of a graft comprising, introducing an effective amount of a nucleic acid encoding an angiogenic agent to the cells of the graft.

2. The method of claim 1, wherein the nucleic acid is contacted prior to transplantation of the graft into a compatible host.

3. The method of claim 1, wherein the graft is tissue.

4. The method of claim 1, wherein the graft is skin.

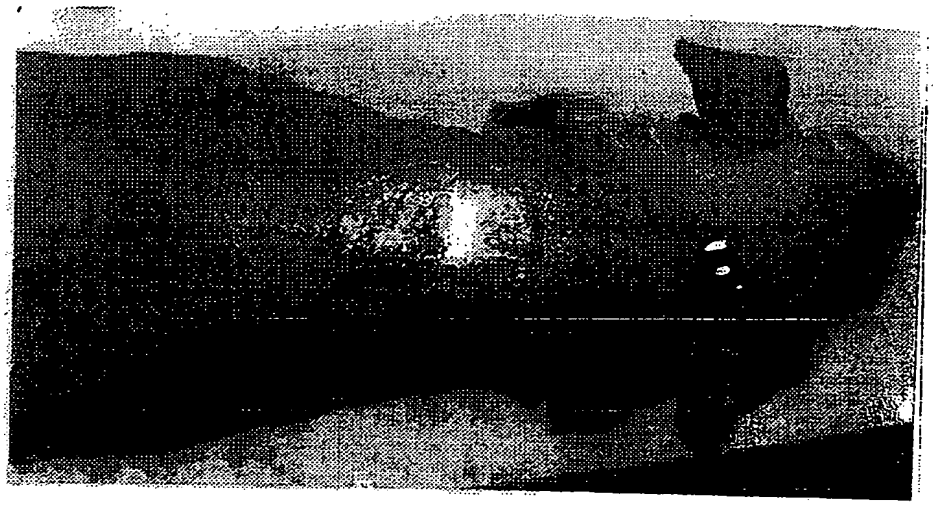

FIG. 1

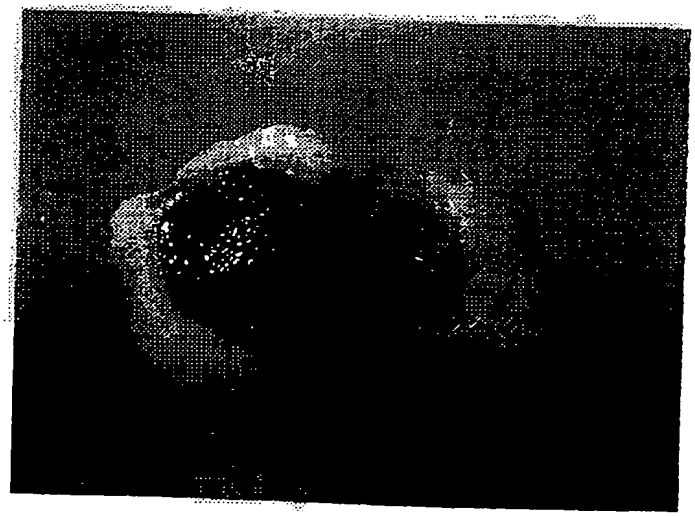

FIG. 2

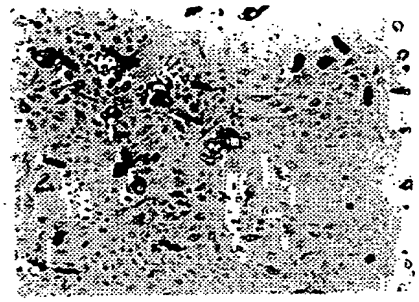

FIG. 3A

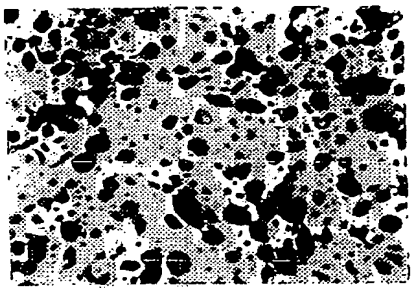

FIG. 3B

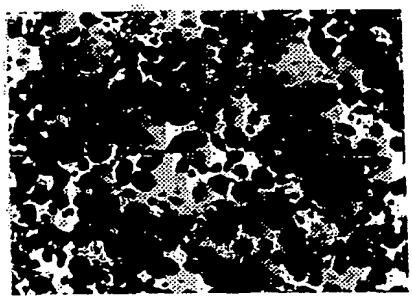

FIG. 3C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15971

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00
US CL :514/44; 424/93.2, 93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 424/93.2, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS and Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NABEL, E. et al. Recombinant Fibroblast Growth Factor-1 Promotes Intimal Hyperplasia and Angiogenesis in Arteries In Vivo. Nature. 29 April 1993, Vol. 362, pages 844-846, see entire document.	1-4
Y	DETMAR, M. et al. Overexpression of Vascular Permeability Factor/Vascular Endothelial Growth Factor and Its Receptors in Psoriasis. Journal of Experimental Medicine. September 1994, Vol. 180, pages 1141-1146, see entire document.	1-4
Y	US 5,639,725 A (O'REILLY et al.) 17 June 1997, col. 4, lines 21-47.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 SEPTEMBER 1998	Date of mailing of the international search report 20 OCT 1998
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